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Rapid Induction of Dendritic Spine Morphogenesis by *trans*-Synaptic EphrinB-EphB Receptor Activation of the Rho-GEF Kalirin

Peter Penzes,[1,2] Alexander Beeser,[3] Jonathan Chernoff,[3] Martin R. Schiller,[4] Betty A. Eipper,[4] Richard E. Mains,[4] and Richard L. Huganir[1,2,*]

[1]Department of Neuroscience and
[2]Howard Hughes Medical Institute
The Johns Hopkins University School of Medicine
725 North Wolfe Street
Baltimore, Maryland 21205
[3]Fox Chase Cancer Center
Philadelphia, Pennsylvania 19111
[4]Department of Neuroscience
University of Connecticut Health Center
263 Farmington Avenue
Farmington, Connecticut 06030

Summary

The morphogenesis of dendritic spines, the major sites of excitatory synaptic transmission in the brain, is important in synaptic development and plasticity. We have identified an ephrinB-EphB receptor *trans*-synaptic signaling pathway which regulates the morphogenesis and maturation of dendritic spines in hippocampal neurons. Activation of the EphB receptor induces translocation of the Rho-GEF kalirin to synapses and activation of Rac1 and its effector PAK. Overexpression of dominant-negative EphB receptor, catalytically inactive kalirin, or dominant-negative Rac1, or inhibition of PAK eliminates ephrin-induced spine development. This novel signal transduction pathway may be critical for the regulation of the actin cytoskeleton controlling spine morphogenesis during development and plasticity.

Introduction

Dendritic spines are thin protrusions emerging from dendrites that are the sites of most excitatory synapses in the CNS. Spines are believed to increase the connectivity of neurons, to provide chemical compartmentalization for calcium and proteins, and to participate in neural computation (Sorra and Harris, 2000; Yuste and Bonhoeffer, 2001). Recent studies have shown that dynamic changes in the structure and shape of spines are important for synaptic function (Harris, 1999; Yuste and Bonhoeffer, 2001).

Spines are very dynamic structures, and their size, shape, and number change during development and adulthood. During development, dendritic protrusions start out as filopodia, which search out contacts with synaptic terminals and then mature into adult spines (Ziv and Smith, 1996). In adults, modulation of the number and shape of spines is associated with synaptic plasticity (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Toni et al., 1999), learning (Comery et al, 1996), and aging, as well as neurological disorders such as mental retardation (Purpura, 1974; Geinisman et al., 1992). However, the molecular mechanisms underlying the initial formation of spines during development and the plasticity of spines in adults are not clear.

We have previously shown that an isoform of the dual Rho-GEF kalirin, containing the first Rac1-GEF domain but lacking the second RhoA-GEF domain, is enriched in spines, regulates spine shape, and may play a critical role in the regulation of spine morphogenesis and plasticity (Penzes et al., 2001). To investigate the extracellular factors and upstream signals that might regulate kalirin activity, we began to examine the effect of known synaptic extracellular ligands on spine structure. Among the extracellular signals tested were the B-type ephrins, membrane-bound ligands localized at excitatory synapses in developing and mature neurons (Buchert et al., 1999; Torres et al., 1998). Ephrins localized in the presynaptic membrane have been suggested to *trans*-synaptically activate their receptors, the EphB receptor tyrosine kinases, in the postsynaptic membrane (Torres et al., 1998; Takasu et al., 2002). Recent work showed that EphB2, by phosphorylating syndecan-2, is able to induce spine morphogenesis in cultured hippocampal neurons (Ethell et al., 2001). In addition, several recent studies have shown that EphB receptor activation by ephrins can regulate clustering of NMDA receptors in the postsynaptic membrane (Dalva et al., 2000; Grunwald et al., 2001) and regulate the calcium channel properties of the NMDA receptor (Takasu et al., 2002). Moreover, generation of *EphB2* mutant mice has demonstrated that ephrinB-EphB2 signaling is important in plasticity and behavior (Grunwald et al., 2001; Henderson et al., 2001).

In this study we show that ephrin-Eph receptor signaling acting through the Rho-GEF kalirin is also involved in the regulation of spine morphogenesis. Treatment of hippocampal neurons with clustered ephrinB1 induced rapid morphogenesis of dendritic spine-like protrusions. These structures had presynaptic contacts and contained key components of functional synapses, such as glutamate receptors and protein components of the postsynaptic density. By overexpressing a kinase-inactive EphB2 receptor, we further showed that the ephrin B1 effect on dendrites is mediated by the kinase activity of EphB2 receptors. EphB2 tyrosine kinase was able to phosphorylate kalirin, and treatment of neurons with clustered ephrinB1 resulted in tyrosine phosphorylation of endogenous kalirin-5 and -7. Ephrin B1 treatment of HEK293 cells expressing kalirin-7 and EphB2 resulted in their coclustering, while treatment of hippocampal neurons with clustered ephrinB1 induced the redistribution of endogeneous kalirin to larger and more synaptic clusters. Overexpression of a GEF-inactive mutant of kalirin, as well as a dominant-negative form of Rac1 blocked the ephrinB effects on dendrites, suggesting that kalirin and Rac1 functioned downstream of EphB2. Furthermore, ephrinB1 treatment induced phosphorylation of PAK, a key target of Rac1, and blocking PAK

*Correspondence: rhuganir@jhmi.edu

activity resulted in blocking the ephrinB effects on spines. Together, our data reveal a novel signaling pathway through which ephrinB and EphB2 receptors regulate dendritic spine morphology.

Results

EphrinB Regulates Dendritic Spine Morphogenesis

To test whether B-type ephrins regulate morphogenesis of dendritic spines, we used 10-day-old hippocampal neurons previously transfected with GFP to visualize their morphology. At this age, dendrites of control neurons were covered with thin, long filopodia-like protrusions (Figure 1A, left). In contrast, a 1 hr treatment of these neurons with clustered ephrinB1 resulted in a dramatic increase in the number and size of dendritic protrusions (Figure 1A, right). On ephrinB1-treated neurons, dendrites were covered with a variety of structures: some were long, thin, protrusions, resembling filopodia (f); some were short and wide protrusions (sw); and many were large protrusions, many had long necks with mushroom-like heads, and are referred to as "spine-like structures" (s) (Figure 1A, right). These results were substantiated by measurements of the average linear density, area, length, and breadth of dendritic protrusions (Figure 1B). We used single factor ANOVA analysis for this and all the other data sets to determine the statistical significance of the differences between groups. The average of spine linear densities for ephrinB-treated neurons (41.18 ± 14.4) was significantly larger than for untreated control neurons (27.26 ± 15.4, $p < 0.0001$). The averages of spine areas (1.94 ± 2.17 versus 0.6 ± 0.55, $p < 0.0001$), lengths (2.52 ± 1.08 versus 1.35 ± 0.7, $p < 0.0001$), and breadths (1.63 ± 0.35 versus 2.19 ± 0.8, $p < 0.001$) for ephrinB-treated neurons were also significantly larger than for untreated control.

At this stage, the majority of synapses in control neurons were shaft synapses (Figure 1D, top, arrows), as previously described (Papa et al., 1995). However, on ephrinB1-treated neurons, almost 50% of the dendritic protrusions (filopodial- and spine-like protrusions) contacted presynaptic terminals, as revealed by synaptophysin staining (Figure 1D, bottom, arrowheads), compared to about 10% in control neurons (Figure 1C, left). Moreover, 30% of the total synaptic contacts were made by large spines (Figure 1C, center), which virtually all contacted presynaptic terminals (Figure 1D, bottom). In addition, the total number of presynaptic terminals increased as a result of ephrinB treatment (Figure 1C, right). The differences between ephrinB treated and control were statistically significant by Student's t test (left, $p < 0.001$; center, $p < 0.001$; right, $p < 0.0003$). These results suggest that the ephrinB1-induced, large spine-like structures had formed synapses. Many such spines contained AMPA receptors and postsynaptic densities, as shown by immunostaining with GluR2 and PSD-95 antibodies (Figure 1E). Together, these data suggest that ephrinB treatment rapidly induced the formation of spines, which were in contact with presynaptic terminals, and contained components of the postsynaptic density including glutamate receptors.

The Kinase Activity of EphB Receptors Is Required for Spine Morphogenesis

EphrinB1 treatment induced phosphorylation of the EphB2 receptor at synaptic sites, as revealed by partial colocalization with synaptophysin (Figure 2A). EphB2 receptors are present (Figure 2B, top) in spines and in large punctate structures in the shafts. Use of an antibody specific for phosphorylated EphB2 demonstrates that ephrinB treatment stimulates the appearance of phosphorylated EphB2 in spines (Figure 2B, bottom). Thus, EphB kinase activity is present at synapses, suggesting that EphB receptors mediate the response to the ephrins. EphB receptors have previously been shown to have kinase-dependent and -independent effects (Wilkinson, 2001). To distinguish between these two possibilities, we cotransfected hippocampal neurons with a kinase-dead EphB2 (EphB2-KD) and GFP (Figure 2C) (Dalva et al., 2000). Exogenous flag-EphB2-KD was targeted to punctate structures along dendrites (data not shown). Overexpression of flag-EphB2-KD reduced the effects of ephrinB1 treatment on spines, resulting in a spine linear density and size comparable to the control neurons, indicating that kinase activity is required for this effect (Figure 2D). As calculated by single factor ANOVA analysis, the average spine linear density of EphB2-KD-expressing neurons (24.4 ± 15.3) was significantly lower than that of ephrinB-treated neurons (41.18 ± 14.4, $p < 0.0001$), but similar to untreated neurons (27.26 ± 15.4, $p = 0.37$). Similarly, the average spine area of EphB2-KD-expressing and ephrinB-treated neurons (1.08 ± 1.28) was significantly lower than that for ephrinB-treated neurons (1.94 ± 2.17, $p < 0.0001$).

Cortical neurons grown at high density were less responsive to ephrinB1 treatment than hippocampal neurons, possibly due to the lower expression levels of EphB2 in cortex than in hippocampus at this age (DIV12) (Henderson et al., 2001). To investigate whether the effects of ephrinB1 were mediated by the EphB2 receptor, we cotransfected cortical neurons with myc-EphB2 and GFP. Exogenous myc-EphB2 was concentrated in punctate structures along dendrites and in dendritic spines, in a similar fashion to endogenous EphB2 (Torres et al., 1998) (Figure 3A). Treatment with clustered ephrinB1 or overexpression of EphB2 without ephrinB1 treatment resulted in little change in spine structure (Figure 3B). However, treatment of cortical neurons overexpressing EphB2 with clustered ephrinB1 resulted in an increased linear density of spines, but no significant change in spine area (Figure 3C). The average spine linear density of EphB2-expressing and ephrinB-treated neurons (53.73 ± 30.1) was higher than that for control untreated neurons (39.5 ± 21.8, $p < 0.001$); the average spine area of EphB2-expressing and ephrinB-treated neurons (1.06 ± 1.08) was similar to control untreated neurons (0.76 ± 0.94, $p = 0.07$). Together, these data demonstrate that B-type ephrins acting on EphB receptors can regulate spine morphogenesis.

Ephrin Treatment Recruits Kalirin to EphB Clusters

Since we had previously seen that the Rho-GEF kalirin regulates spine structure, we wanted to test whether

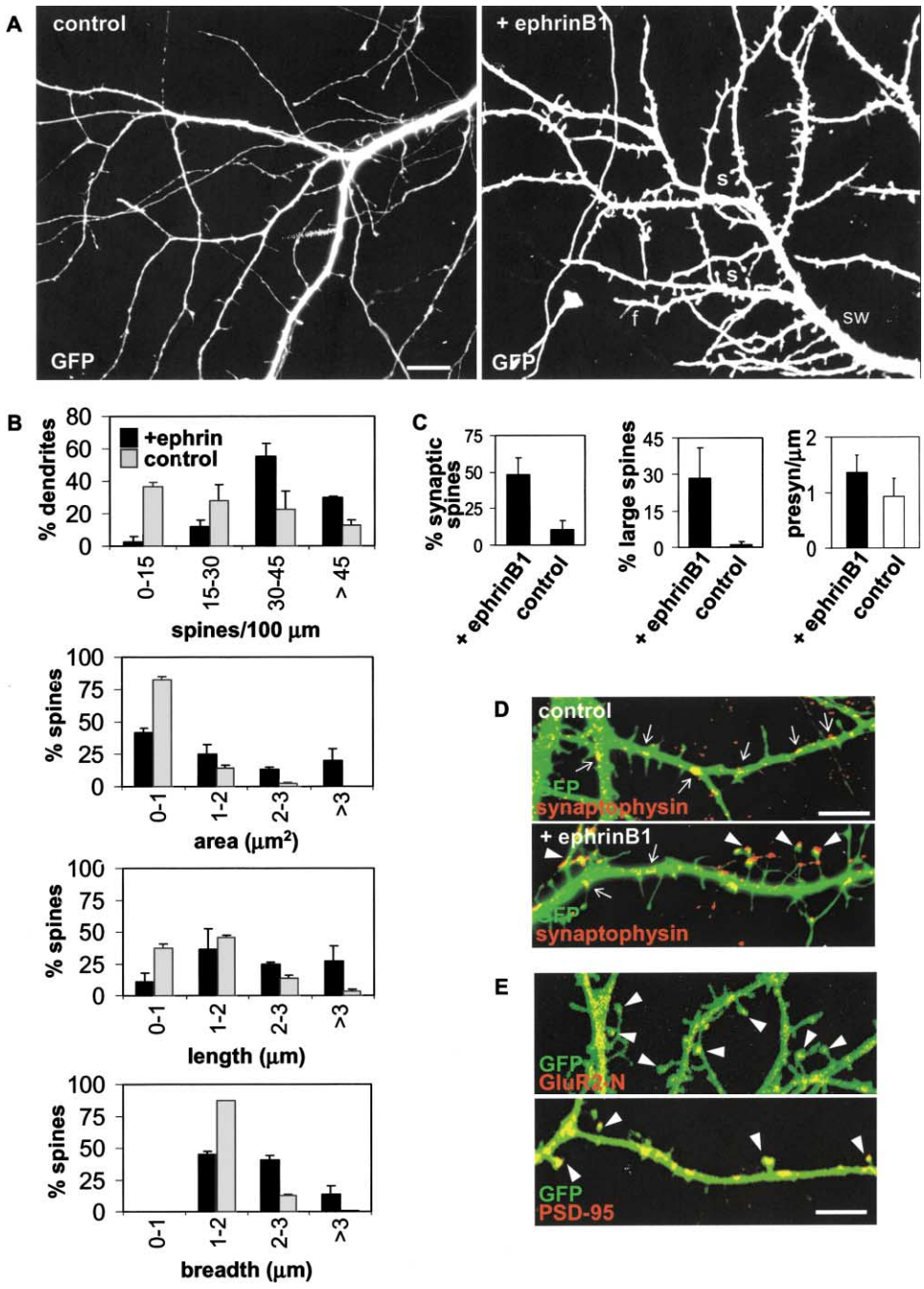

Figure 1. EphrinB1 Stimulates Dendritic Spine Morphogenesis in Cultured Hippocampal Neurons

(A) Hippocampal neurons, 10 days in vitro (DIV10), transfected with GFP 7 days earlier, were left untreated (control, left), or treated with clustered ephrinB1 for 1 hr (right), fixed, and immunostained for GFP.

(B) Quantification of the effects of ephrinB1 treatment (dark bars) on spine linear density and size, as reflected by spine area, length, and maximum breadth; untreated, light bars.

(C) Quantification of synaptic contacts and morphologies of ephrinB-induced and control spines; left, percent relative to total protrusions which make contact with presynaptic terminals; center, percent relative to total large protrusions (area larger than 2 μm^2) which make contact with presynaptic terminals; right, linear density of presynaptic sites.

(D) Analysis of presynaptic contacts made by dendritic protrusions in control and ephrinB1-treated neurons. Neurons were immunostained for GFP and synaptophysin; arrows indicate shaft synapses; arrowheads indicate spine synapses.

(E) Analysis of postsynaptic proteins in ephrinB1 treatment-induced spines; neurons were immunostained for GFP and PSD-95 or GluR2. Arrowheads indicate presence of postsynaptic proteins in spines. Scale bars: 10 μm (A); 5 μm (D) and (E). Bars represent averages of data from two experiments, and error bars are standard deviations.

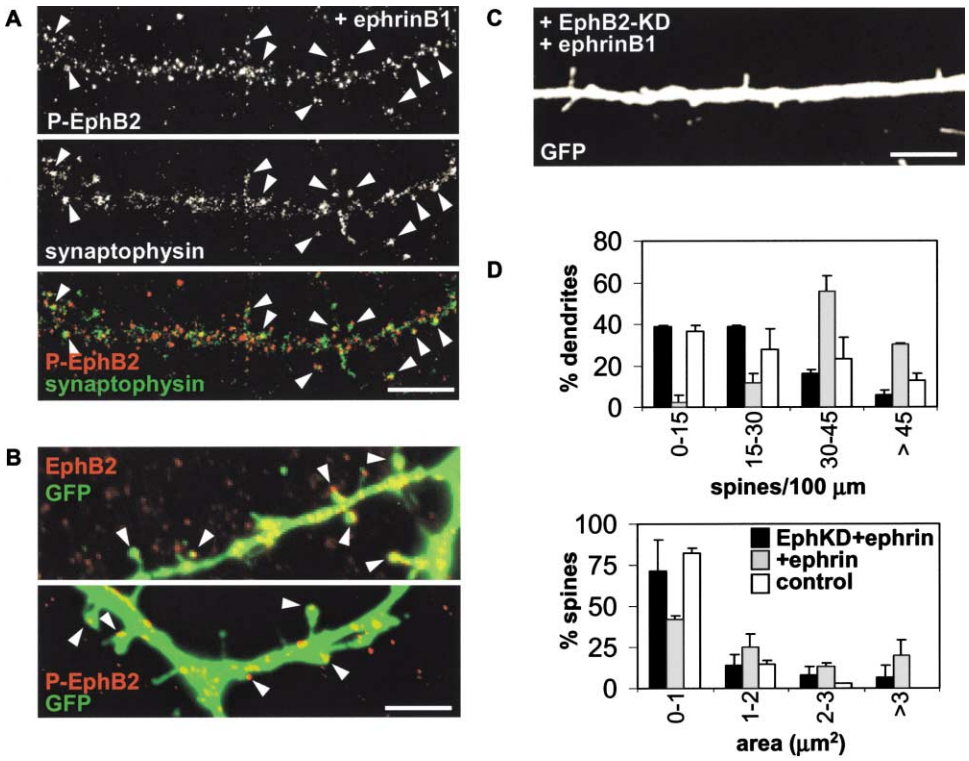

Figure 2. Role of EphB2 in Spine Morphogenesis in Cultured Hippocampal Neurons

(A) EphB2 is phosphorylated at synapses (arrowheads). EphrinB1-treated hippocampal neurons were stained with antibodies for phospho-EphB2 (P-EphB2) and synaptophysin.

(B) EphB2 is present and is phosphorylated in spines (arrowheads). Hippocampal neurons transfected with GFP were treated with clustered ephrinB1, fixed, and stained with antibodies for EphB2 (top) or phospho-EphB2 (P-EphB2, bottom) and GFP.

(C) Transfection of kinase-dead EphB2 (EphB2-KD) abolishes the effects of ephrinB1 on spine morphogenesis in hippocampal neurons. Hippocampal neurons (DIV10) cultured at low density, previously transfected with GFP and Flag-EphB2-KD, were treated with clustered ephrinB1 for 2 hr and stained with GFP and flag antibodies.

(D) Quantification of the effects of ephrinB1 treatment of EphB2-KD overexpressing neurons (dark bars) on spine linear density and size (area); ephrinB1-treated (gray bars, from Figure 1); untreated cells (light bars, from Figure 1). Scale bars: 5 μm (A) and (C), 2 μm (B). Bars represent averages of data from two experiments, and error bars are standard deviations

the ephrinB effects could be mediated by regulation of kalirin function. We first tested whether kalirin was a substrate for the EphB2 receptor tyrosine kinase by co-transfecting HEK293 cells with the isoforms of kalirin present at synapses, kalirin-7, and kalirin-5 with or without EphB2. We immunoprecipitated kalirin proteins from lysates of ephrinB1-treated cells (Penzes et al., 2000, 2001), and detected their tyrosine phosphorylation state by Western blotting with a phosphotyrosine-specific antibody (pY20) (Figure 4A). In this heterologous cell system, the EphB receptor induced tyrosine phosphorylation of kalirin-7 and kalirin-5. To confirm this result in neurons, we treated cortical neurons with clustered ephrinB1 and immunoprecipitated kalirin proteins from neuronal homogenates using a pan-kalirin antibody (kalspec) (Penzes et al., 2000) (Figure 4B). We then examined the tyrosine phosphorylation of immunoprecipitated kalirin proteins. EphrinB1 treatment resulted in an increased tyrosine phosphorylation of kalirin-5 and kalirin-7, while the immunoprecipitated protein amounts were similar.

It was possible that phosphorylation of kalirin by EphB2 resulted in a change in either the GEF activity or in the subcellular localization of kalirin. To test the first possibility, we transfected HEK293T cells with kalirin-7 and EphB2, alone or together, and measured the activation of endogenous Rac1 by an affinity binding assay (Figure 4C). While expression of EphB2 alone induced a slight increase in Rac1 activation, as shown by the binding of Rac1 to the Rac binding domain of PAK1 (PBD), coexpression of kalirin-7 and EphB2 did not affect the level of Rac1 activation. Treatment with clustered ephrinB1 of double-transfected cells did not have any effect on GEF activity. These results suggest that phosphorylation of kalirin by EphB2 may not regulate its GEF activity.

Many GEFs are recruited to the plasma membrane after activation of their upstream signal transduction pathways (Michiels et al., 1997). To examine whether EphB receptor activation regulated the subcellular distribution of kalirin-7, we transfected HEK293T cells with myc-tagged kalirin-7 and Flag-tagged EphB2, alone or in combination (Figure 4D). When expressed alone, EphB2 was perinuclear and associated with the plasma membrane, sometimes forming small clusters, while kalirin-7 expressed alone was diffusely distributed in the cytosol. Treatment with clustered ephrinB1 of cells expressing EphB2 or kinase-dead EphB2 (EphB2-KD) alone in-

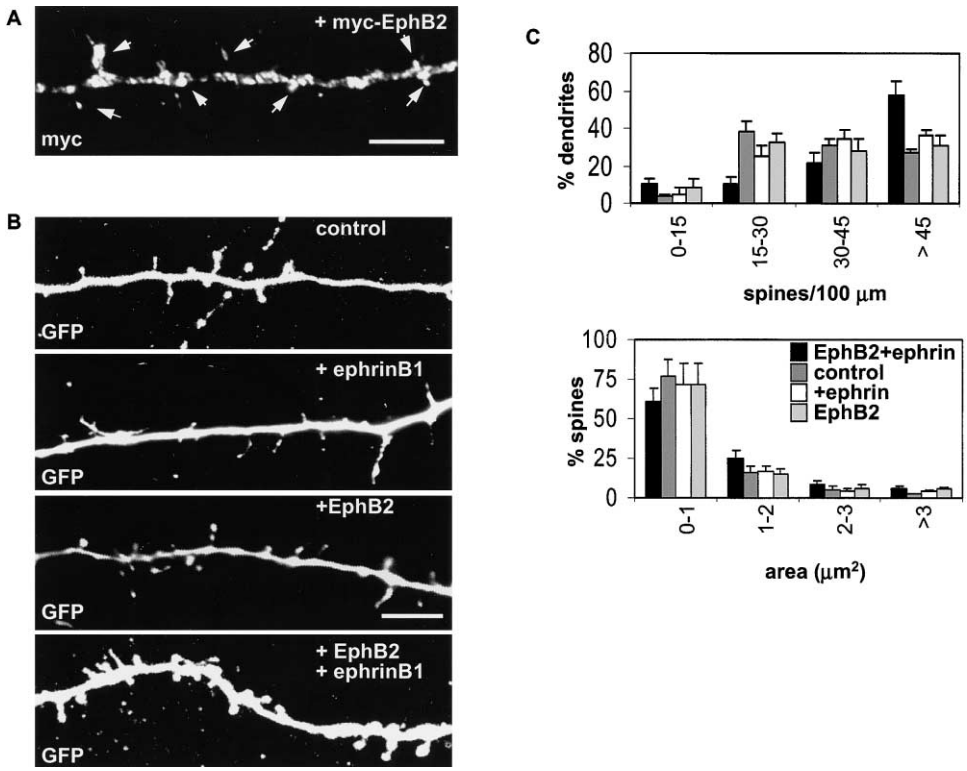

Figure 3. Role of EphB2 in Spine Morphogenesis in Cultured Cortical Neurons

(A) Localization of exogenous myc-EphB2 to dendritic spines (arrows). High-density cultures of cortical neurons were transfected with myc-EphB2 and GFP, and 7 days later (DIV12), were stained with GFP and myc antibodies.

(B) High-magnification images comparing the spines on cortical neurons (DIV12): control neurons transfected with GFP only and untreated (top), neurons treated with clustered ephrinB1 but transfected only with GFP (second), neurons transfected with GFP and EphB2 but untreated (third), and neurons transfected with EphB2 and treated with ephrinB1 (bottom).

(C) High-density cultures of cortical neurons: quantification of the effects on spine linear density and size of EphB2 overexpression and ephrinB1 treatment (dark bars), compared to untreated controls (dark gray bars), neurons treated with ephrinB1 but transfected only with GFP (white bars), and transfected with GFP and EphB2, but untreated (light gray bars). Scale bars, 5 μm. Bars represent averages of data from two experiments, and error bars are standard deviations.

duced clustering of EphB2 or EphB2-KD, while it did not induce clustering of kalirin-7 expressed alone. Coexpression of EphB2 with kalirin-7, without ephrin treatment, resulted in a slightly patchier distribution of kalirin-7 (arrows). However, treatment of these doubly transfected cells with clustered ephrinB1 resulted in a dramatic coclustering of kalirin-7 and EphB2, resulting in large membrane-associated clusters. To examine the role of the kinase activity of EphB2 in clustering of kalirin-7, we cotransfected cells with kinase-dead EphB2 (EphB2-KD) and kalirin-7. Treatment of these cells with clustered ephrinB1 resulted in clustering of EphB2-KD, but not of kalirin. These results suggest that binding of ephrinB1 to EphB2 resulted in EphB2 clustering, and recruitment of cytoplasmic kalirin-7 to these membrane-associated clusters in a kinase-dependent manner.

EphrinB Action on Spines Is Mediated by Kalirin

To test whether activation of endogenous EphB resulted in redistribution of endogenous kalirin-5/7, we treated hippocampal neurons with clustered ephrinB1 and visualized kalirin-5/7 with an isoform-specific antibody (Penzes et al., 2000) (Figure 5A). EphrinB1 treatment induced a significant redistribution of kalirin-5/7 to large clusters along the dendrites and in spines (Figures 5A, top, and 5B, left), in contrast to the localization of kalirin-5/7 to very small puncta, with relatively few large clusters in spines, in control neurons at this stage of development (Figures 5A, bottom, and 5B, right). Many of these new kalirin-5/7 clusters were synaptic, as shown by colocalization with synaptophysin (Figure 5B). Quantification of the percentage of overlap between kalirin and synaptophysin staining, as a measure of synaptic localization, showed that indeed ephrin-treated neurons had a higher fraction of kalirin localized to synapses than untreated control neurons (Figure 5C, left). Quantification of the kalirin cluster sizes, reflected by their areas, demonstrated that in ephrin-treated neurons kalirin clusters were larger than in control neurons (Figure 5C, right). The differences between ephrinB-treated neurons and controls were statistically significant by Student's t test (Figure 5C; left, $p < 0.001$; right, $p < 0.001$). Together, these data indicate that EphB activation by ephrinB1 induces recruitment of kalirin-5/7 to dendritic clusters and spines, where kalirin may function downstream of EphB2 in regulating spine morphogenesis. To test this hypothesis, we cotransfected hippocampal neurons

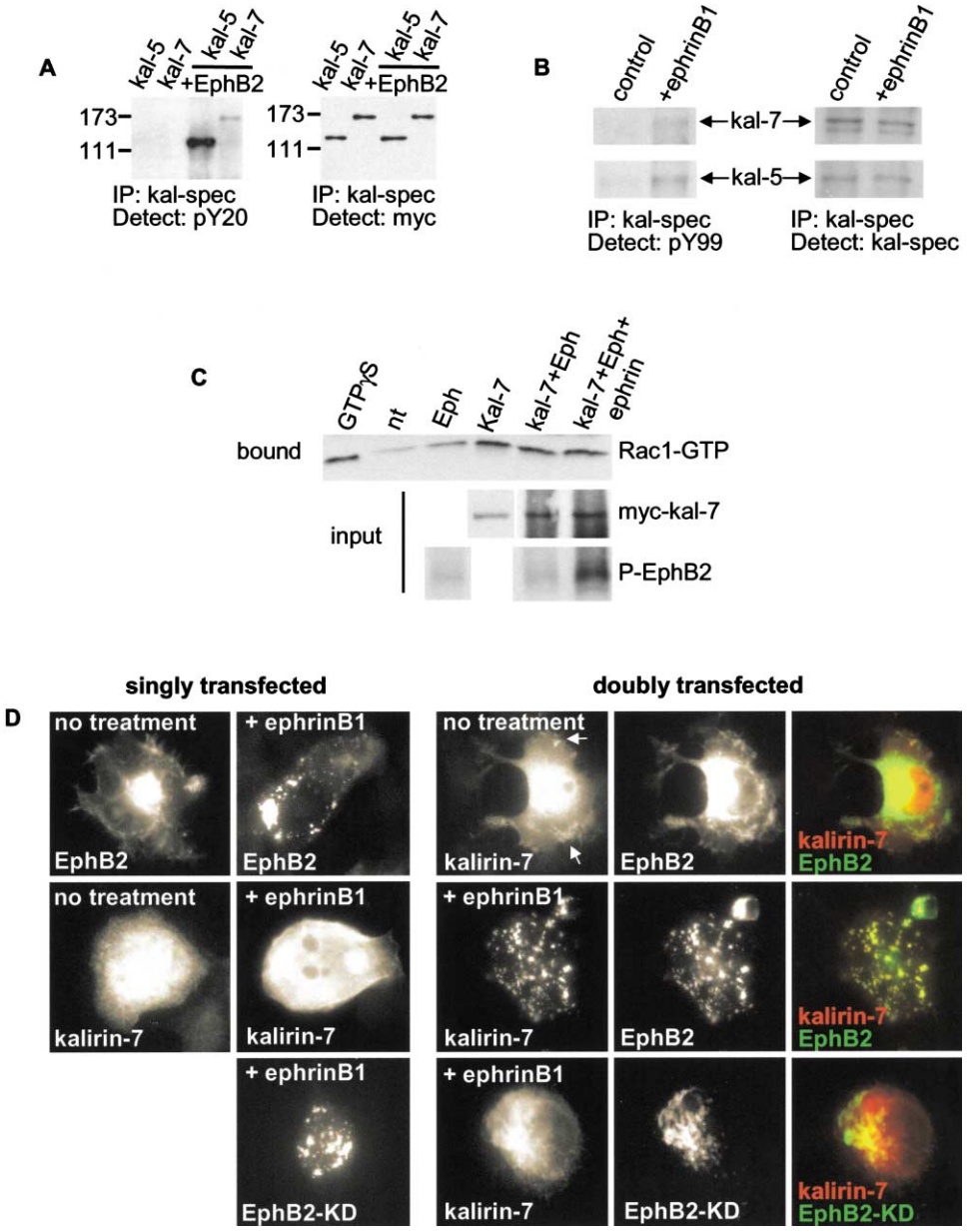

Figure 4. EphB Phosphorylates and Clusters Kalirin

(A) Phosphorylation of kalirin by EphB2. HEK 293 cells were transfected with myc-kalirin-5 or myc-kalirin-7, alone or with Flag-EphB2; cells were treated with clustered ephrin-B1, and kalirin proteins were immunoprecipitated with a kalirin antibody. Tyrosine phosphorylation of kalirin proteins was visualized by Western blotting with a phosphotyrosine antibody (pY20) (left). Amounts of immunoprecipitated kalirin protein were normalized by Western blotting with the myc antibody (right).

(B) Phosphorylation of kalirin in neurons. Cortical neurons (DIV21) were either left untreated or treated with clustered ephrinB1, and kalirin proteins were immunoprecipitated with a pan-kalirin antibody (kal-spec). Tyrosine phosphorylation of kalirin proteins was visualized by Western blotting with a phosphotyrosine antibody (pY99) (left). Immunoprecipitated kalirin proteins were visualized with the kal-spec antibody (right).

(C) Rac1 activation assay: EphB2 does not affect the GEF activity of kalirin-7. HEK 293 cells were transfected with myc-kalirin-7 or myc-EphB2, alone or together; cells were left untreated or treated with clustered ephrinB1. Activated Rac1 protein was isolated by binding to the Rac1 binding domain of PAK1 (PBD) crosslinked to resin. Bound Rac1, expressed myc-kalirin-7, and phospho-EphB2 (P-EphB2) were visualized by Western blotting with Rac1, myc, and P-EphB2 antibodies, respectively.

(D) EphrinB1 induces coclustering of EphB2 and kalirin-7. HEK 293 cells plated onto slides were transfected with myc-kalirin-7, Flag-EphB2, or Flag-EphB2-KD (kinease dead), alone or together; cells were left untreated or treated with clustered ephrin-B1. Transfected proteins were visualized using the Flag and kalirin antibodies.

with a GEF-inactive form of kalirin-7 (Kal7-mut) along with GFP (Figure 5D). Upon treatment with clustered ephrinB1, neurons expressing kal7-mut had fewer and smaller dendritic spines than nontransfected neurons treated with ephrinB1 (Figures 5D and 5E) similar to the untreated control neurons (Figures 1A and 1B). The

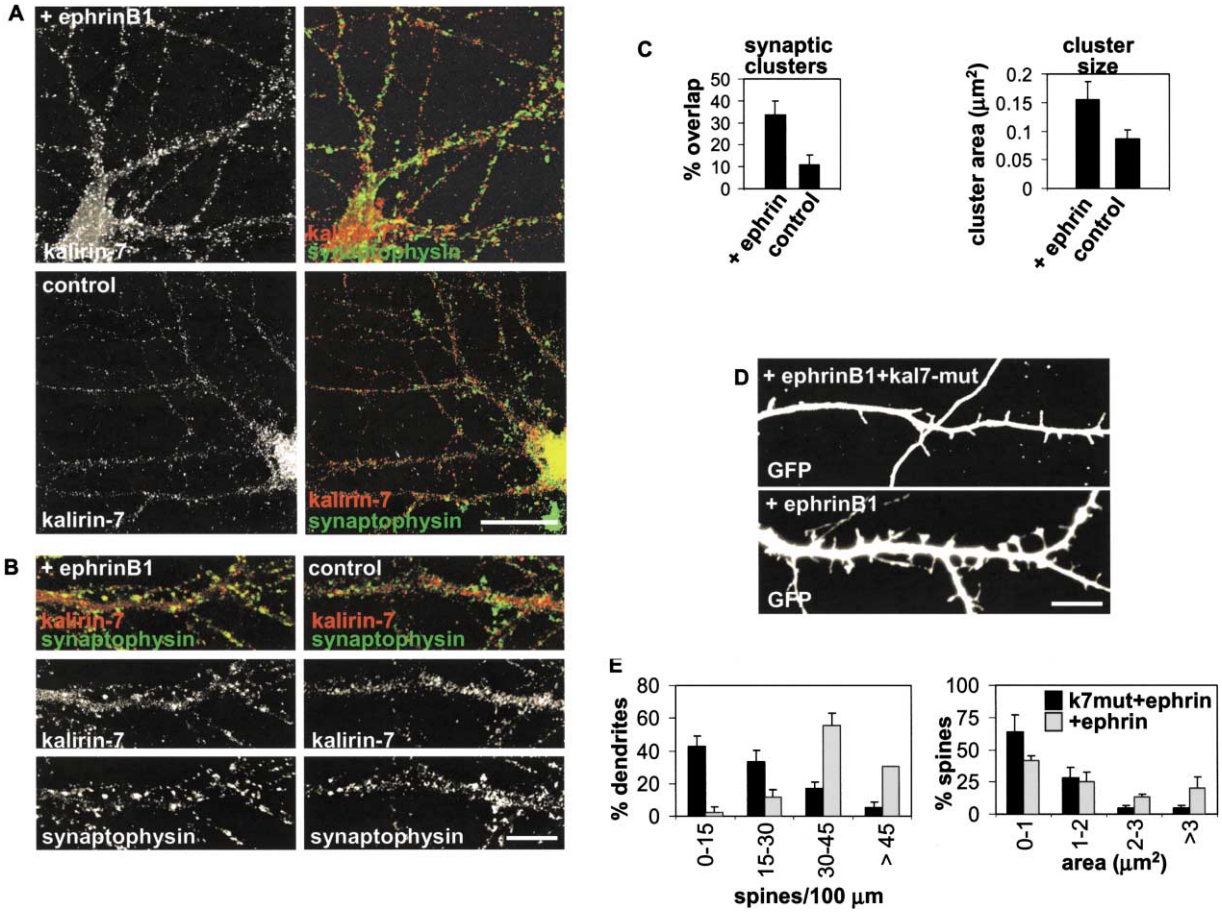

Figure 5. EphrinB and EphB Signaling in Spines Is Mediated by Kalirin

(A) EphrinB1 induces synaptic clustering of kalirin-7 in hippocampal neurons. Hippocampal neurons cultured at low density (DIV15) were treated with clustered ephrinB1 (top), or untreated (bottom), and were fixed and stained with antibodies for kalirin-7 and synaptophysin.

(B) Higher magnification images of control (untreated) and ephrinB1-treated neurons.

(C) Quantification of the ephrinB1 effect on kalirin-7 distribution. Left, synaptic localization of kalirin-7, as reflected by the percent of overlap of kalirin-7 puncta with synaptophysin for individual dendrites. Right, size of kalirin-7 clusters, as reflected by the areas of individual kalirin-7 puncta measured using the "Colocalization" module of the Metamorph software.

(D) A GEF mutant kalirin-7 (kal7-mut) abolishes the effects of ephrinB1 treatment on spine morphogenesis. Cultured hippocampal neurons (DIV10), previously transfected with myc-kal7-mut and GFP (top) or GFP alone (bottom), were treated with clustered ephrinB1 for 2 hr and stained for myc and GFP.

(E) Quantification of the effects of ephrinB1 treatment of kal7-mut expressing neurons (k7mut, dark bars) on spine linear density and size (area); ephrinB1-treated controls, light bars (from Figure 1). Bars represent averages of data from four coverslips, and error bars are standard deviations. Scale bars: 10 μm (A); 5 μm (B) and (C).

average spine linear density of kal-7mut-expressing neurons (22.7 ± 14.4) was significantly lower than that for ephrinB-treated controls (41.18 ± 14.4, $p < 0.0001$); the average spine area of k7mut-expressing neurons (0.95 ± 1.05) was lower than that for ephrinB-treated controls (1.94 ± 2.17, $p < 0.001$). Thus, kal7-mut interfered with the downstream signaling by the EphB2 receptor, indicating that kalirin may mediate EphB2 signaling.

Rac1 and PAK Mediate EphrinB Action on Spines

Kalirin activates the Rho GTPase Rac1 (Penzes et al., 2001a), which has been shown to regulate spine morphogenesis (Nakayama et al., 2000). The effects of the GEF1 domain of kalirin on process extension and retraction in cortical neurons were blocked by dominant-negative Rac1 (Penzes et al., 2001b). Therefore, we examined the involvement of Rac1 in the ephrinB1-mediated response by transfecting hippocampal neurons with a dominant-negative form of Rac1 (Rac1-N17) along with GFP, prior to treatment with ephrinB1 (Figure 6A). Dominant-negative Rac1 abolished the effects of ephrinB1 on spine morphogenesis (Figure 6B). The average spine linear density of Rac1-N17-expressing neurons (12.19 ± 7.1) was significantly lower than for ephrinB-treated controls (41.18 ± 14.4, $p < 0.0001$), while the average spine area of Rac1-N17-expressing neurons (0.46 ± 0.62) was significantly lower than that for ephrinB-treated controls (1.94 ± 2.17, $p < 0.001$) or untreated controls (0.6 ± 0.55, $p < 0.001$).

To further investigate the signal transduction pathway, we examined the role of the Rac1 effector p21-

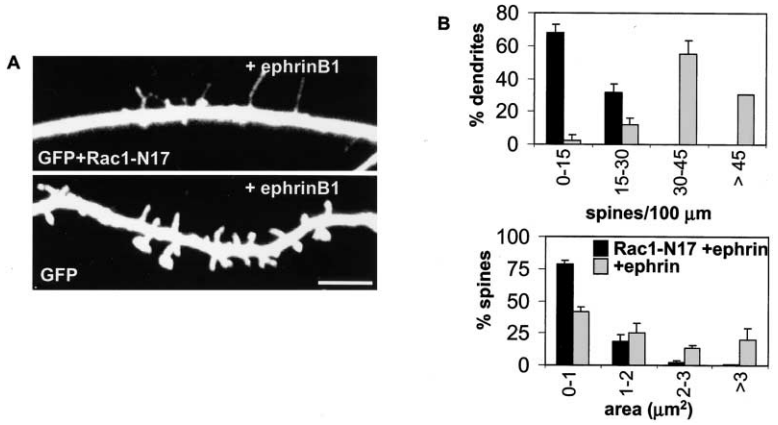

Figure 6. Regulation of Spine Morphogenesis by EphrinB1 Is Mediated by Rac1

(A) Dominant-negative Rac1 (Rac1-N17) (top) abolishes the effect of ephrinB1 on spine morphogenesis, as compared with ephrinB1-treated control neurons (bottom). Hippocampal neurons cultured in low density, previously transfected with Rac1-N17 and GFP, were treated with clustered ephrinB1 for 2 hr and immunostained for GFP and Rac1.

(B) Quantification of the effects of ephrinB1 treatment on spine linear density and size (area) in hippocampal neurons overexpressing Rac1-N17 (dark bars); ephrinB1-treated controls (light bars). Scale bar, 5 μm. Bars represent averages of data from two experiments, and error bars are standard deviations.

activated kinase PAK. Several PAK proteins are expressed in the brain, and previous studies have shown that some of the effects of Rac1 on the cytoskeleton are mediated by PAK (Bagrodia and Cerione, 1999). In addition, genetic analysis in *Drosophila* has shown that *PAK1* is genetically associated with *dTrio*, the fly ortholog of *kalirin*, in the pathway through which *dTrio* affects axon growth and guidance (Newsome et al., 2000). Previous studies have shown that binding of activated Rac1 to PAK induces PAK autophosphorylation, which strongly correlates with its activation (Sells et al., 2000). To test whether ephrinB treatment induced activation of PAKs, we used an antibody detecting autophosphorylated PAK (P-PAK) (Sells et al., 2000) (Figure 7A). In addition, this experiment can be regarded as a way to visualize endogenous Rac1 activation. Treatment of hippocampal neurons with clustered ephrinB1 induced a dramatic increase in the number and size of clusters stained with the P-PAK antibody. This effect was confirmed by Western analysis with the P-PAK antibody of extracts of 4-week-old high-density cortical neurons treated with ephrinB1 (Figure 7B). Moreover, in hippocampal neurons, ephrinB1 treatment induced activation of PAK at synapses, as shown by P-PAK immunostaining coincident with synaptophysin (Figure 7C).

To test whether kalirin-7 was required for ephrinB1-induced PAK phosphorylation, we examined the effect of overexpressing the GEF inactive kal7-mut in hippocampal neurons on the ability of clustered ephrinB1 to induce phosphorylation of PAK. Therefore, we transfected DIV7 hippocampal neurons with myc-kal7-mut, and 2 days later we treated the neurons with clustered ephrinB1 for 2 hr, followed by fixation and immunostaining for myc and P-PAK. While ephrinB1 treatment induced an increased phosphorylation of PAK in nontransfected neurons, in neurons expressing kal7-mut, the level of P-PAK was visibly reduced compared to adjacent nontransfected neurons (Figure 7D). Quantification of the ratios of P-PAK fluorescence intensities to total cell areas of nontransfected control neurons relative to the same ratios for neurons expressing myc-kal7-mut confirmed this observation (Figure 7E). The difference between kal7-mut and control were statistically significant by Student's t test ($p < 0.0014$). Overexpression of GFP alone did not affect the ability of clustered ephrinB1 to induce PAK phosphorylation, suggesting that overexpression of any protein does not interfere with PAK phosphorylation (data not shown).

PAKs phosphorylate proteins involved in regulating the actin cytoskeleton and gene expression (Bagrodia and Cerione, 1999; Wilkinson, 2001). To test whether PAK was an essential downstream component of ephrinB signaling in spine morphogenesis, we treated GFP-transfected hippocampal neurons with a fusion protein of the PAK1 inhibitory domain (PID) fused with the cell-penetrating peptide (TAT-PID) along with ephrinB1 (Vocero-Akbani et al., 2001; Zhao et al., 1998) (Figure 7F). These neurons exhibited a reduction in the number and size of spines, compared to the ephrinB1-treated neurons (Figure 7G), while also showing a reduced phosphorylation level of PAK, confirming its inhibition by PID (Figure 7F). The average spine linear density of TAT-PID-treated neurons (25.66 ± 8.4) was significantly lower than that of ephrinB-treated neurons (41.18 ± 14.4, $p < 0.0001$), but similar to untreated controls (27.26 ± 15.4, $p = 0.55$); the average of spine areas for TAT-PID-treated neurons (0.43 ± 0.42) was lower than for ephrinB-treated controls (1.94 ± 2.17, $p < 0.0001$). Together, these data demonstrate that Rac1 and PAK are key downstream components of ephrinB regulation of spine morphogenesis.

Discussion

We previously reported that kalirin-7 is a regulator of spine morphogenesis (Penzes et al., 2001). Here we identified an upstream regulator of kalirin-7, as well as key components of the downstream pathway mediating its effect on spines. Previous studies have demonstrated the role of ephrins and Eph receptors in the inhibition of axon growth (Wilkinson, 2001; Shamah et al., 2001). In this study we extend recent reports that Eph receptors play a role in synaptic function (Dalva et al., 2000; Ethell et al., 2001) and identify an ephrinB-EphB receptor *trans*-synaptic signal transduction pathway that regulates spine morphogenesis (Figure 8). Postsynaptic EphB receptors may be activated by the clustering of presynaptic ephrinB during development or synaptic plasticity. Subsequently, activated EphB2, by phosphorylating kalirin or other intermediate substrates, may induce the recruitment of kalirin to signaling complexes in dendrites and spines, increasing the local concentra-

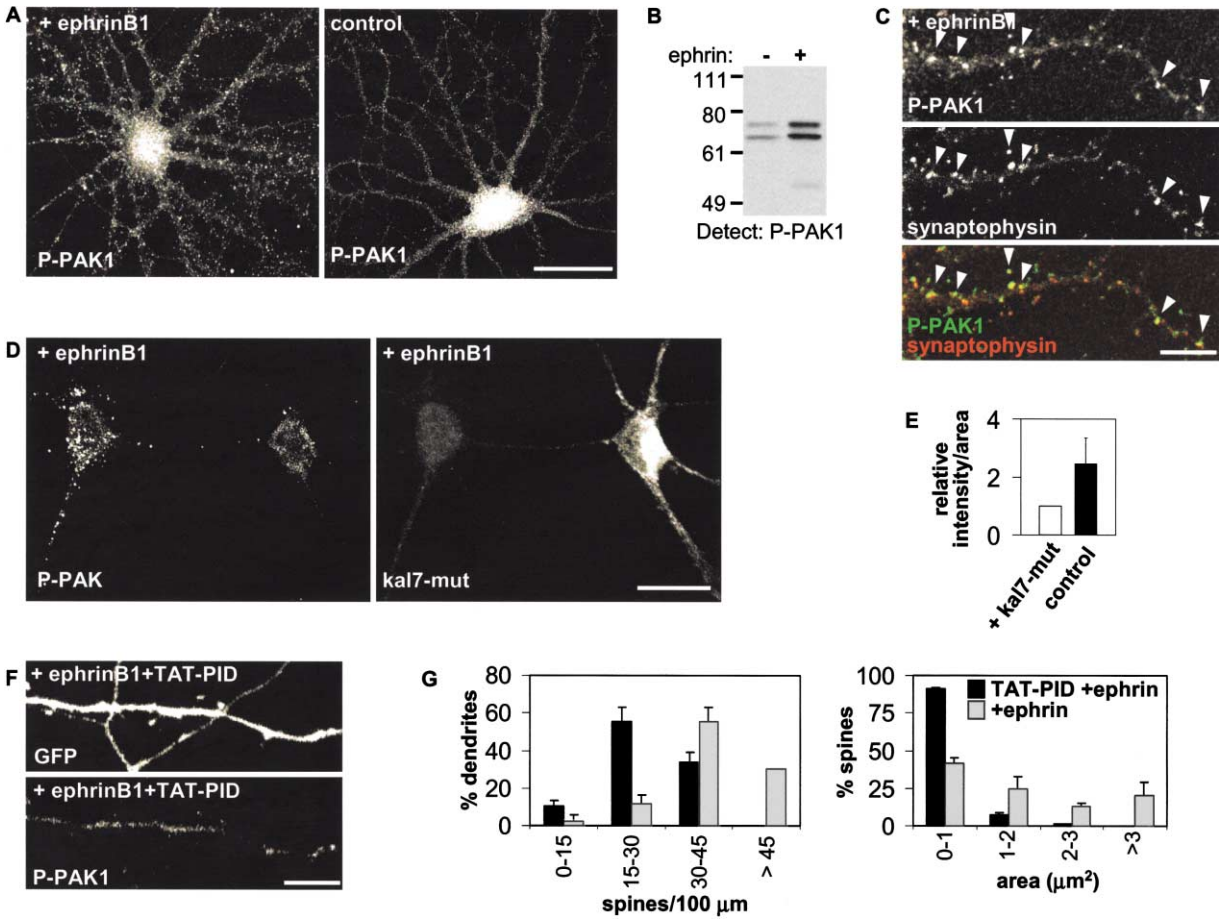

Figure 7. Regulation of Spine Morphogenesis by EphrinB1 Is Mediated by PAK

(A) Treatment with clustered ephrinB1 induces activation of PAK in hippocampal neurons (DIV15). Activated PAK was visualized by immunostaining with anti-phospho-PAK antibody (P-PAK).

(B) Western blot showing activation of PAK proteins in cortical neurons (DIV21) treated with clustered ephrinB1.

(C) Activation of PAK by clustered ephrinB1 occurs at synapses in hippocampal neurons, shown by staining with antibodies for P-PAK and synaptophysin; arrowheads indicate synaptic P-PAK.

(D) PAK phosphorylation induced by clustered ephrinB1 is blocked by overexpression of mutant kalirin-7 (kal7-mut) in hippocampal neurons (DIV13).

(E) Quantification of the ratios of P-PAK fluorescence intensities to total cell areas of nontransfected control neurons relative to the same ratios for neurons expressing myc-kal7-mut. Neurons were outlined manually to create regions; region areas and P-PAK fluorescence intensities inside the regions were measured using Metamorph. For each neuron, the ratio between P-PAK fluorescence and area was calculated; for each picture, the intensity/area ratios of each cell were divided by that of the cell expressing kal7-mut, and the average of these ratios was plotted as relative intensity/area.

(F) EphrinB1-induced spine morphogenesis is abolished by PAK-inhibitory domain (PID). Hippocampal neurons expressing GFP (DIV10) were incubated with the cell-permeant PAK-inhibiting peptide (TAT-PID) for 2 hr and clustered ephrinB1 for 1 hr, and stained with antibodies for GFP (top) and P-PAK (bottom).

(G) Quantification of the effects on spine linear density and size (area) of TAT-PID on ephrinB1-treated neurons (dark bars); ephrinB1-treated controls, light bars (from Figure 1). Scale bars: 10 μm, (A) and (D); 5 μm, (C) and (F). Bars represent averages of data from two experiments, and error bars are standard deviations.

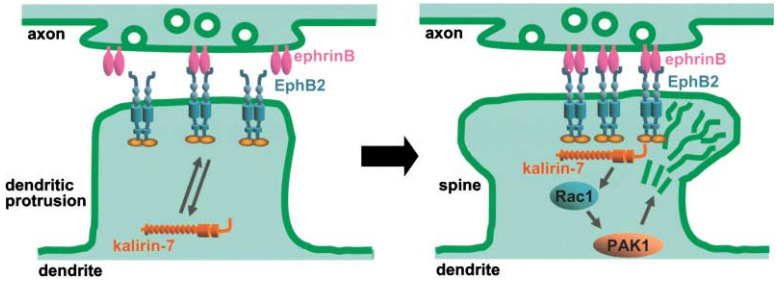

Figure 8. Model of Regulation of Spine Morphogenesis by EphrinB, Kalirin, and PAK

Clustering of presynaptic ephrinB induces clustering and activation of postsynaptic EphB receptors. These, in turn, induce synaptic recruitment of kalirin, resulting in local activation of Rac1 and PAK. Actin rearrangements regulated by these pathways may result in spine morphogenesis.

tion of kalirin, resulting in increased local Rac1 activation. This may then induce local activation of PAK, which triggers cascades of events, resulting in actin rearrangements and spine morphogenesis (Bagrodia and Cerione, 1999; Wilkinson, 2001). Due to the similarity of PAK forms, we cannot determine the individual form involved. This novel *trans*-synaptic signaling pathway may allow coordination between the pre- and postsynaptic side of synapses to regulate the generation of new spines during development and plasticity.

A number of recent reports established a role for EphB2 receptors in synaptic plasticity. Mutant mouse studies implicated EphB2 in synaptic plasticity, in a manner independent of its kinase activity (Grunwald et al., 2001; Henderson et al., 2001). Activation of the EphB2 receptors induces clustering of NMDA receptors in young neurons, important for the formation of new synapses (Dalva et al., 2000; Grunwald et al., 2001). In addition, activated EphB2 induces Src-mediated phosphorylation of the NMDA receptor, resulting in the potentiation of its calcium channel properties and expression of calcium-induced immediate-early genes (Takasu et al., 2002). Another recent study has reported that phosphorylation by EphB2 of the synaptic protein syndecan-2 may play a role in the regulation of spine structure (Ethell et al., 2001). Interestingly, the EphB2 mutant mice did not show dramatic alterations of dendritic structure (Grunwald et al., 2001; Henderson et al., 2001). This could potentially be due to the compensatory effect by other members of the EphB family. Together, these data suggest that ephrinB-EphB2 may regulate synaptogenesis in two distinct ways. Through its extracellular domain, activated EphB2 induces NMDA receptor clustering (Dalva et al., 2000), while activation of its kinase domain regulates NMDA receptor channel function and spine morphogenesis.

During development, it is necessary to coordinate accurately the formation and location of presynaptic active zones with those of the postsynaptic structures. This could be achieved by signaling from presynaptic ephrinB, clustered at active zones on axons, to activate postsynaptic EphB2, resulting in synaptogenesis on the apposing dendrites. Even in mature neurons, dendritic spines are very dynamic structures (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Toni et al., 1999; Fischer et al., 1998; van Rossum and Hanisch, 1999), and recent studies have demonstrated that LTP induces morphological changes in spines, which may contribute to plasticity in adult neurons (Engert and Bonhoeffer, 1999; Maletic-Savatic et al., 1999; Lendvai et al., 2000; Toni et al., 1999). The rapid and dramatic effect of ephrinB on spine maturation suggests that ephrinB-EphB2 signaling may be a key component in the regulation of spine morphogenesis during plasticity. Other extracellular signals have been shown to regulate spine morphogenesis, such as K^+ depolarization (Wu et al., 2001), glutamate action on NMDA receptors (Maletic-Savatic et al., 1999), and BDNF (Murphy et al., 1998; Shimada et al., 1998). It is possible that kalirin mediates the intracellular effects of these signals as well.

Knowledge of the molecular components of the signal transduction pathways that control the structure, function, and plasticity of synapses is important in understanding the processes of learning and memory, but also of disease processes such as mental retardation and aging-related cognitive impairments. Several proteins have been implicated in the regulation of spine formation, suggesting a general scheme of regulation of spine morphogenesis. Cell-surface receptors, such as EphB2 (Ethell et al., 2001), TrkB (Shimada et al., 1998), and NMDA receptors (Maletic-Savatic et al., 1999) are activated by extracellular signals: adhesion signals, neurotrophins, and neurotransmitters. These receptors relay these signals to signal transduction proteins, such as SPAR (Pak et al., 2001), MAPK (Wu et al., 2001), Rac1 and RhoA (Nakayama et al., 2000; Tashiro et al., 2000), and Kalirin-7 (Penzes et al., 2001), which regulate rearrangements of the actin cytoskeleton. These signaling proteins are anchored to the PSD by scaffolding and actin binding proteins, such as PSD-95 (El-Husseini et al., 2000), Shank (Sala et al., 2001), Homer (Sala et al., 2001), spinophilin (Feng et al., 2000), and drebrin (Hayashi and Shirao, 1999). Although several players have been identified, the picture remains largely fragmentary, in that most of these players are not connected in pathways. In this paper we show that the ephrinB→EphB→kalirin→Rac1→PAK→actin pathway is critically important for the regulation of spine morphogenesis.

Experimental Procedures

Antibodies and Reagents

Anti-phospho-PAK1 antibody was used at dilutions of 1:2000 for Westerns and 1:400 for immunostaining (Sells et al., 2000). Anti-phospho-EphB2 was a gift of Drs. Michael Greenberg and Matthew Dalva (Harvard Medical School, Cambridge, MA) and was used at 1:1000 (Dalva et al., 2000). Anti-EphB2 antibodies were gifts from Drs. Michael Greenberg, Matthew Dalva, and Jeanine Zieg (Harvard Medical School) and Dr. Anthony Pawson (University of Toronto, Toronto, ON, Canada) and both were used at 1:1000 (Binns et al., 2000; Dalva et al., 2000). Anti-kalirin-7 antibody (JH2959) was used at 1:1000 for Westerns and 1:400 for immunostaining; anti-kalirin-spectrin antibody (JH2582) was used at 1:1000 for Westerns (Penzes et al., 2000). Anti-Flag monoclonal antibody was from Sigma and was used at 1:2000 for Westerns and 1:5000 for immunostaining. EphrinB1-Fc was from R&D Research. Anti-human Fc goat polyclonal antibody was from Sigma. Anti-Rac1 antibody was from Upstate Biotechnology and was used at 1:1000 for Western blotting. Monoclonal anti-GFP antibody was from Molecular Probes, and a polyclonal anti-GFP antibody was generated in the Huganir lab. Phospho-tyrosine antibodies pY99 (used at 1:1000) and pY20 (used at 1:250) were from Santa Cruz Biotechnology and Transduction Laboratories, respectively. DNAs: Flag-EphB2 and Flag-EphB2-KD were a gift from Drs. Matthew Dalva and Michael Greenberg (Dalva et al., 2000).

Neuronal Cultures, Transfections, and Treatments

Low- and medium-density hippocampal neurons and high-density cortical neurons were cultured as described (Penzes et al., 2001a). Hippocampal neurons were transfected at DIV4 with the calcium phosphate method (Penzes et al., 2001) and kept 1 week thereafter. Cortical neurons were transfected at DIV5 with calcium phosphate. GEF mutant kalirin-7 (kal-mut) was generated by PCR mutagenesis of residues Asn1415 and Asp1416 to Ala within the DH domain of kalirin-7. The mutant protein was found to lack any detectable GEF activity using the Rac1 activation assay kit (Upstate Biotechnology). The DNA fragment containing the mutations was subcloned into pEAK-His-myc-kalirin-7, using the PacI and NsiI restriction sites. For treatments, ephrinB1-Fc (50 μg/ml) was incubated for 30 min in culture medium with 0.1 μg/ml anti-human Fc antibody (Dalva et al., 2000) and added to cells for 2 hr at 37°C. TAT-PID (fusion of TAT cell-permeant peptide with PAK-inhibitory domain) (32 μg/ml)

was added to neurons for 2 hr, in glial-conditioned medium (Vocero-Akbani et al., 2001).

Morphometric Measurements

Neurons cultured on coverslips were fixed for 20 min in 3.7% formaldehyde and permeabilized and blocked in PBS containing 10% normal donkey serum and 0.1% Triton X-100. Antibodies were added in PBS with 2% normal donkey serum, and mounted slides were examined with a Zeiss 510 confocal microscope. In double transfection experiments, neurons were immunostained with antibodies for GFP along with antibodies for the tag of the transfected protein (for Flag-EphB2-KD, myc for myc-EphB2 and myc-kal7-mut, or Rac1 for Rac1-N17). All healthy neurons expressing both proteins from each coverslip were imaged and quantified. Z-stacked images were analyzed using the Metamorph software (Universal Imaging Corporation) as follows. Images of GFP-positive neurons, collected with the 63× objective, were thresholded in Metamorph to exactly outline spines. The spines were selected using the "regions" tool and analyzed using the "Integrated Morphometry" feature. Next, the length of the analyzed region on a particular dendrite was measured. Colocalization was analyzed using the "Colocalization" module; images to be compared were taken with the same exposure parameters and thresholded equally. "Large spines" were defined as spines with areas over 2 μm^2. In each experiment, between 400 and 600 spines from 5 to 10 neurons were analyzed per condition. Two to four experiments per condition were pooled for quantification. Data were reused in separate figures.

Cell Cultures, Transfections, and Western Blotting

HEK293T cells cultured in MEM with 10% fetal bovine serum were transfected with Lipofectamine 2000 (Invitrogen) with 2 μg DNA/well in 30 mm plastic dishes. After 12 hr, cells were treated with clustered ephrinB1 for 1 hr, harvested, and homogenized in 1 ml PBS with 1% triton X-100, with protease and phosphatase inhibitors. Extracts were incubated with 5 μl/tube of JH2582 for 1 hr at 4°C and with 40 μl Protein-A Sepharose (Pharmacia) for 1 hr. Pellets were washed 3× with 1 ml of homogenization buffer and analyzed by Western blotting with JH2582, P-EphB2, and phosphotyrosine antibody pY20 (Transduction Laboratories). Cortical neurons were treated with clustered ephrinB1 for 2 hr and harvested on ice into 0.5 ml PBS with 1% triton X-100 with protease and phosphatase inhibitors. Extracts were centrifuged at 10,000 × g for 10 min at 4°C, and supernatants were analyzed by Western blotting with anti-phospho-PAK1 antibody. For Rac1 activation assay, HEK293T cells cultured and transfected as above, were serum starved overnight in Opti-MEM (GIBCO). After 16 hr, cells were treated with clustered ephrinB1 for 1 hr, and Rac1 activation was measured using the Rac1 activation assay kit (Upstate Biotechnology). Briefly, extracts (1 mg/ml) were incubated with 10 μl/tube of PBD resin for 1 hr at 4°C. Pellets were washed 3×; inputs and pellets were analyzed by Western blotting with myc, P-EphB2, and Rac1 antibodies.

For EphB2-kalirin clustering assays, HEK293T cells were cultured in chamber slides (Nalge Nunc) and transfected as above. After 48 hr, cells were either left untreated or treated with clustered ephrinB1 for 1 hr, fixed for 20 min in 3.7% formaldehyde in PBS, permeabilzed, and blocked in PBS containing 10% normal donkey serum and 0.1% Triton X-100. Antibodies were added in PBS with 2% normal donkey serum.

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